**TITLE**

ANTIBODIES TO NUCLEUS PULPOSUS IN DISC HERNIATION, DIAGNOSTIC KIT, MEDICAL PREPARATIONS AND TREATMENT

**DESCRIPTION**

Technical field

The present invention relates the use of serum antibodies for the diagnosis and treatment of disc herniation with resulting nerve root pain in the cervical and lumbar spine such as sciatica.

The object of the present invention is to obtain improved methods in diagnosis and treatment of nerve root pain such as sciatica and other radiculopathies related to disc herniation in the cervical or lumbar spine.

Background of the invention

The exact pathophysiological mechanisms leading to sciatica in relation to herniation of intervertebral discs are not fully understood. Recently it was demonstrated that the nucleus pulposus (the viscous component of the intervertebral disc that leaks out into the spinal canal in case of disc herniation) may induce structural and functional changes in the adjacent nerve root (1-14). Also, it has been shown that nerve roots experimentally exposed to nucleus pulposus become sensitive to mechanical deformation thereby producing pain (8,13). Certain pro-inflammatory cytokines, produced by the nucleus pulposus cells, have been defined as being responsible for inducing these effects (10). However, there is both clinical and experimental evidence which may suggest that also immunologic mechanisms may be present to a certain extent. It has been suggested that, since the nucleus pulposus is secluded from the immune-system from birth, being a non-vascularized tissue, the immune system has not regarded the nucleus pulposus as "self" during early embryonic stages, but would instead consider the nucleus pulposus as "non-self" later in life (15-22). At disc herniation, possible antigens in the nucleus pulposus might thus be presented to the immune system and there would be an auto-immune reaction induced towards these antigens. The reaction would mainly involve the nucleus pulposus, but the substances might also induce changes in the adjacent nerve roots secondary to this reaction. These suggested substances would most likely be the same pro-inflammatory cytokines as previously being defined as

inducing nerve root injury. There is reason to believe that such mechanisms also relates to radiculopathies in the upper extremities due to disc herniation in the cervical spine. However, no one has previously been able to demonstrate the presence of antibodies in serum towards the nucleus pulposus of the same individual.

In order to isolate and show the presence of antibodies towards nucleus pulposus cells the following experiments and tests were conducted.

Material and methods

1) Culture of nucleus pulposus cells:

One pig weighing 26 kg was anaesthetized with an intra muscular injection of 20 mg/kg body weight of Ketalar$^{R}$ (ketamine 50 mg/ml, Parke-Davis, Morris Plains, NJ) and an intravenous injection of 4 mg/kg body weight of Hypnodil$^{R}$ (methomidate chloride 50 mg/ml, AB Leo, Helsingborg, Sweden) and 0,1 mg/kg body weight of Stresnil$^{R}$ (azaperon, 2 mg/ml, Janssen Pharmaceutica, Beerse, Belgium).

Approximately 20 ml of blood were collected and allowed to coagulate at room temperature. It was then centrifuged and the supernatant (serum) was stored at 80°C in a refrigerator.

After induction of anaesthesia, the pig was killed by an overdose of potassium chloride. The lumbar and lower part of the thoracic spine was removed en bloc. The spine was cleansed from muscles and tendons. Under sterile conditions the discs were incised and the nucleus pulposus was harvested. The nucleus pulposus (NP) was washed once in Ham's F12 medium (Gibco BRL, Paisley, Scotland).The NP from discs were placed in a test tube with Ham's F12 medium and centrifuged. The remaining pellet was dissolved in 6 ml of Ham's F12 with 3 ml of trypsine 2.5 % in a 75 cm$^2$ culture flask for 30 minutes at 37°C. Then 6 ml of Ham's F12 with 12 mg of collagenase (Sigma Cat. No. C9407) were added. After 3.5 hrs at 37°C the content of the culture flask was transferred to a test tube and centrifuged. The separated NP-cell pellets were suspended in DMEM/F12 1:1 medium (Gibco BRL, Paisley, Scotland) supplemented with 1% L-glutamine (200 mM, Gibco BRL, Paisley, Scotland), 50 µg/ml gentamycine sulphate (Gibco BRL, Paisley, Scotland) and 10% foetal calf serum

(FCS, Gibco BRL, Paisley, Scotland). Fungizone 2 µg/ml and α-ascorbic acid 50 µg/ml was added. The cells were cultured in 25 $cm^2$ flasks (Costar, Cambridge, MA), at 37°C and 5% $CO_2$ in air for 3-4 weeks . After 2 weeks the cells were transferred to 4-chamber polystyrene vessel tissue culture treated glass slides (Becton Dickinson Labware, Franklin Lakes, NJ). Following 3 days of culture the slides were used for the assessment as will be described below.

2) Culture of fibroblasts

A 2x2 cm big piece of the skin was harvested at the same time as the nucleus pulposus under sterile conditions. The dermis of the skin was cut in small pieces and put in spinner bottles with 10 ml of collagenase solution (0.8 mg/ml, Sigma Chemical, St. Louis, MO, in Ham's F12 medium) for 90 minutes in 37°C water bath. The separated fibroblasts were centrifuged and transferred to 75 $cm^2$ tissue culture flasks (Costar, Cambridge, MA), with DMEM/F12 1:1 medium supplemented as above for NP-cells.

3) Pretreatment of the serum

The cultured fibroblasts were liberated from the culture flasks by treatment of 0.125% trypsine solution (Gibco BRL, Paisley, Scotland) and added to the serum. The addition of fibroblasts was performed in order to eliminate the risk that antibodies in the serum which non-specifically would bind to cultured cells, would be applied to the nucleus pulposus cells. The test-tube was centrifuged and the supernatant collected (serum with remaining antibodies).

4). Assessment of the presence of antibodies in serum towards autologous nucleus pulposus cells

The culture slides with the cultured nucleus pulposus cells were fixed in acetone for 10 minutes and then dried in air. The slides were washed twice for 5 minutes in PBS (Phosphate Buffered Saline, Life Technologies Ltd., Paisley, Scotland) The slides were then treated with 0.3% H2O2 (Sigma Chemical, St. Louis, MO) for 30 minutes and then washed twice for 5 minutes in PBS. The slides were then exposed to standard freeze-dried milk (5% in PBS) for 30 minutes to block irrelevant antigens, and then washed twice for 5 minutes in PBS.

The cultured NP-cells were exposed to

a) one drop of the pretreated serum,

b) one drop of the pretreated serum diluted by PBS 1:40; or

c) not in serum at all, and

incubated for 1 hr at room temperature, and then washed twice for 5 minutes in PBS. The culture slides were then incubated with the secondary antibody (Peroxidase-Conjugated Rabbit Anti-Swine immunoglobulin, Code No. P164, Dako A/S, Glostrup, Denmark) for 30 minutes, and then washed twice for 5 minutes in PBS. The slides were finally developed with DAB (3,3'-diaminobenzidine, 10 mg in 5 ml PBS, and 17 µl $H_2O_2$ (3%), Sigma Chemical, St. Louis, MO) for 2 minutes, and then washed twice for 5 minutes in PBS. The specimens were dehydrated in a series of alcohol-dilutions and assessed by light microscopy.

Results

a) Pretreated serum

There was a clear staining of the cell membranes of the nucleus pulposus cells and also of the nuclei of the cells. This indicates the presence of specific antibodies towards the nucleus pulposus cells in serum from the same individual (autologous).

b) Pretreated serum at 1:40

There was a similar staining of the cells as for the concentrated serum, although not as pronounced.

c) Nucleus pulposus cells not exposed to serum

There was no staining of the cells and the cells were difficult to distinguish on the culture slides. This suggests that the secondary antibody (rabbit:anti-swine-immunoglobulin) did not non-specifically bind to the pig nucleus pulposus cells and that the staining of a) and b) was the result of the addition of specific antibodies from the serum.

Conclusion and comments

The following conclusion can be made from the present experiment:

- There are antibodies present in serum that specifically bind to nucleus pulposus cells of the

same individual

The following comments can be made from the present experiment

- From this study it can not be recognized if the antibodies are readily available in high concentrations in serum or if there had been an immunization to the nucleus pulposus in the pig. If there are antibodies already present in the serum, the antigen may be a potent antigen, comparable for instance to the MHC (Major Histocompatibility Complex)antigens.
- Regardless of the nature of the antigen one can suspect that the levels in serum may increase the levels of these antibodies in case of disc herniation and sciatica and therefore used as a diagnostic tool.
- At present the diagnosis of sciatica is made by patient history and radiologic findings. However, since it is known that almost 30% of the population without any complaints of sciatica also have disc herniations at radiological examinations, the radiologic diagnosis is less valuable (23-25). It has been suggested that disc herniations can be can be either active (symptoms) or inactive (no symptoms). Based on the findings in the present study it is assumed that an active disc herniation is related to inflammatory and immunologic changes, whereas the inactive disc herniation is a mere protrusion of disc tissue without triggering of the immune system. The lack of immunologic reaction might be based on either the nucleus pulposus still being isolated from the epidural space by remaining membranes or a less developed immunoreactivity of the patient, alternatively lack of sensitizing antigens in the disc cells.

The present invention can thus be used in the form of an antigen containing diagnostic kit for diagnosing disc herniation, in particular disc herniation leading to sciatica. Further the effects of serum antibodies towards the nucleus pulposus cells (NP-antibodies) can be neutralized in three ways. First the NP-antibodies can be inactivated by administering a specific antibody for such serum antibodies, an anti-antibody. Secondly, the effects of the NP-antibodies can be inhibited by administering a substance that is similar to the NP-antibody, a false antibody, and binds to the antigen in the nucleus pulposus in stead of the antibody, which false antibody is able to bind to and block the antigen in such a way that an immunological reaction is inhibited. Thirdly, soluble antigens corresponding to the NP-antibodies can be administered, thereby blocking the effects of the NP-antibodies. In such

ways the action of the NP-antibodies can be blocked since the NP-antibodies are prevented from binding to its antigen.

The compounds of the invention can be administered in a variety of dosage forms, e.g, orally, in the form of tablets, capsules, sugar or film coated tablets, liquid solutions; rectally, in the form of suppositories; parenterally, e.g., intramuscularly or by intravenous injection or infusion. The therapeutic regimen for the different clinical syndromes must be adapted to the type of pathology taken in to account, as usual, also the route of administration, the form in which the compound is administered and age, weight, and condition of the subject involved.

The oral route is employed, in general, for all conditions, requiring such compounds. In emergency cases preference is given to intravenous injection. For these purposes the compounds of the invention can be administered orally at doses ranging from about 20 to about 1500 mg/day. Of course, these dosage regimens may be adjusted to provide the optimal therapeutic response.

The nature of the pharmaceutical composition containing the compounds of the invention in association with pharmaceutically acceptable carriers or diluents will, of course, depend upon the desired route of administration. The composition may be formulated in the conventional manner with the usual ingredients. For example, the compounds of the invention may be administered in the form of aqueous or oily solutions or suspensions, tablets, pills, gelatine capsules (hard or soft ones)syrups, drops or suppositories.

Thus for oral administration, the pharmaceutical compositions containing the compounds of the invention are preferably tablets, pills or gelatine capsules, which contain the active substance together with diluents, such as lactose, dextrose, sucrose, mannitol, sorbitol, cellulose; lubricants, e.g., silica, talc, stearic acid, magnesium or calcium stearate, and/or polyethylene glycols; or they may also contain binders, such as starches, gelatine, methyl cellulose, carboxymethylcellulose, gum arabic, tragacanth, polyvinylpyrrolidone; disaggregating agents such as starches, alginic acid, alginates, sodium starch glycolate, microcrystalline cellulose; effervescing agents such a carbonates and acids; dyestoffs;

sweeteners; wetting agents, such as lecithin, polysorbates, laurylsulphates; and in general non-toxic and pharmaceutically inert substances used in the formulation of pharmaceutical compositions. The mentioned pharmaceutical compositions may be manufactured in known manners, e.g., by means of mixing, granulating, tableting, sugar-coating or film-coating processes. In the case film providing compounds can be selected to provide release in the right place in the intestinal tract with regard to absorption and maximum effect. Thus pH-dependent film formers can be used to allow absorption in the intestines as such, whereby different phthalate are normally used or acrylic acid/methacrylic acid derivatives and polymers.

The liquid dispersions for oral administration may be e.g., syrups, emulsion, and suspensions.

The syrups may contain as carrier, e.g., saccharose, or saccharose with glycerine and/or mannitol and/or sorbitol.

Suspensions and emulsions may contain as carrier, e.g., a natural gum, such as gum arabic, xanthan gum, agar, sodium alginate, pectin, methyl cellulose, carboxymethylcellulose, polyvinyl alcohol.

The suspension or solutions for intramuscular injections may contain together with the active compound, a pharmaceutically acceptable carrier, such as e.g., sterile water, olive oil, ethyl oleate, glycols,, e.g., propylene glycol, and if so desired, a suitable amount of lidocaine hydrochloride. Adjuvants for trigging the injection effect can be added as well.

The solutions for intravenous injection or infusion may contain as carrier, e.g., sterile water, or preferably, a sterile isotonic saline solution, as well as adjuvants used in the field of injection of active compounds.

The suppositories may contain together with the active compound, a pharmaceutically acceptable carrier, e.g., cocoa-butter polyethylene glycol, a polyethylene sorbitan fatty acid ester surfactant or lecithin.

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